

# Killing of *Caenorhabditis elegans* by *Cryptococcus neoformans* as a model of yeast pathogenesis

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We found that the well-studied nematode *Caenorhabditis elegans* can use various yeasts, including *Cryptococcus laurentii* and *Cryptococcus kuetzingii*, as a sole source of food, producing similar brood sizes compared with growth on its usual laboratory food source *Escherichia coli* OP50. *C. elegans* grown on these yeasts had a life span similar to (*C. laurentii*) or longer than (*C. kuetzingii*) those fed on *E. coli*. However, the human pathogenic yeast *Cryptococcus neoformans* killed *C. elegans*, and the *C. neoformans* polysaccharide capsule as well as several *C. neoformans* genes previously shown to be involved in mammalian virulence were also shown to play a role in *C. elegans* killing. These included genes associated with signal transduction pathways (*GPA1*, *PKA1*, *PKR1*, and *RAS1*), laccase production (*LAC1*), and the  $\alpha$  mating type. *C. neoformans* adenine auxotrophs, which are less virulent in mammals, were also less virulent in *C. elegans*. These results support the model that mammalian pathogenesis of *C. neoformans* may be a consequence of adaptations that have evolved during the interaction of *C. neoformans* with environmental predators such as free-living nematodes and amoebae and suggest that *C. elegans* can be used as a simple model host in which *C. neoformans* pathogenesis can be readily studied.

Studies of microbial pathogenesis in nonvertebrate hosts during the past decade have resulted in important insights into the molecular mechanisms of microbial pathogenesis and host defense. It is now apparent that many of the same bacterial virulence factors are involved in pathogenesis in evolutionarily disparate hosts, including plants, nematodes, and mammals (1–15). The nematode worm *Caenorhabditis elegans* has proven to be a particularly facile host for studying bacterial pathogenesis. For example, both Gram-negative and Gram-positive human bacterial pathogens kill *C. elegans*, bacterial virulence factors important for mammalian pathogenesis have been shown to enhance the rate at which *C. elegans* are killed by these pathogens, and bacterial mutant libraries can be readily screened by using a *C. elegans* killing assay for avirulent clones. *C. elegans* can also be readily used to study the host innate immune response to microbial infection (16, 17). *C. elegans* feed on bacteria and the *C. elegans* bacterial killing assay simply involves transferring worms from their normal laboratory food, *Escherichia coli* strain OP50, to a lawn of the pathogen of interest growing on agar medium.

In contrast to bacterial pathogens, *C. elegans* has not been reported as a host for studying human fungal pathogens. Recently Steenbergen *et al.* (18) reported the use of the free-living amoebae *Acanthamoeba castellanii* as a model for the study of survival strategies used by the human opportunistic fungal pathogen *Cryptococcus neoformans* after ingestion by macrophages. Steenbergen *et al.* found that *C. neoformans* was phagocytosed by *A. castellanii*, and that once inside, *C. neoformans* replicated, eventually killing the amoebae. The process was remarkably similar to that seen in mammalian macrophages (19). An acapsular strain of *C. neoformans* did not survive when incubated with *A. castellanii* and a phospholipase mutant exhibited a decreased replication rate in amoebae, similar to results obtained in macrophages (18). These observations suggested

that cryptococcal characteristics that contribute to mammalian virulence also promote fungal survival in free-living amoebae.

Given the successful use of *C. elegans* and *A. castellanii* to study bacterial and fungal pathogenesis, respectively, and because fungal infections are significant causes of human morbidity and mortality, especially among immunocompromised patients (20–26), we sought to develop a fungal–*C. elegans* model pathogenesis system. The increasing number of serious fungal infections, the paucity of new antifungal agents, and the likelihood of the emergence of drug resistance in fungi all contribute to a pressing need for new model systems to study the mechanisms of fungal virulence. However, the ability of *C. elegans* to use yeasts as a food source, and the susceptibility of *C. elegans* to killing by yeasts that are pathogenic to animals and humans, has not been previously reported. Indeed, because of the small size of *C. elegans* and the relatively large size of fungal cells compared with bacterial cells, it was not apparent that a fungal–*C. elegans* model for the study of pathogenesis could be developed.

To determine whether *C. elegans* could consume fungi as a food source and whether human fungal pathogens could kill *C. elegans*, we initially chose to test free-living basidiomycetous yeasts belonging to the genus *Cryptococcus* (27) that presumably interact with nematodes in natural habitats. One reason for selecting cryptococcal species for these experiments was that they form transparent lawns on various growth media, thereby allowing us to easily monitor the survival of *C. elegans* by using a dissecting microscope. *C. laurentii* and *C. kuetzingii* are two generally nonpathogenic cryptococcal species that were used as controls for varieties of the pathogenic species *C. neoformans*. *C. neoformans* is heterothallic with two mating types, *MATa* and *MAT $\alpha$*  and a variety of serotypes (28), and it has been used as a model for the study of fungal pathogenesis. Despite frequent environmental exposure to *C. neoformans*, immunologically intact individuals uncommonly develop clinical disease. However, the incidence of infection rises dramatically in patients with impaired T-cell immunity (26, 29). Study of the pathogenic mechanisms of *C. neoformans* has been enhanced substantially by the development of transformation protocols, homologous recombination for genetic manipulations, and reproducible animal models (30–34). The most important *C. neoformans* virulence factors identified so far include the polysaccharide capsule (30, 35), laccase (an enzyme essential for melanin production) (18, 36–40), the  $\alpha$  allele of the mating type locus (41, 42), and at least two signal transduction cascades (42–47).

In this paper, we show that *C. elegans* can feed on cryptococci, that *C. neoformans* but not *C. laurentii* or *C. kuetzingii* infects and kills *C. elegans*, and that killing by *C. neoformans* depends on a number of virulence genes that are also important in mammalian

Abbreviations: ATCC, American Type Culture Collection; BHI, brain heart infusion; NGM, nematode growth medium; PKA, protein kinase A.

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**Table 1. Yeast strains used and their interaction with *C. elegans***

<i>C. neoformans</i> strains (ref.)	Relevant characteristics or phenotype	LT <sub>50</sub> for <i>C. elegans</i> killing ( <i>P</i> value compared to parent strain, when relevant)
H99 ATCC #208821 (48)	Serotype A; clinical isolate; produces laccase; genome sequence being determined	5.5–7.0 days
H99 <i>gpa1</i> (42)	<i>GPA1</i> encodes a G-protein alpha subunit homolog; attenuated in mammalian models	>10 days ( <i>P</i> < 0.0001)
H99 <i>gpa1</i> + <i>GPA1</i> (42)	Complementation of the <i>gpa1</i> mutant with wild-type <i>GPA1</i> restored virulence in rabbits	5.0 days (no difference)
H99 <i>pka1</i> (44)	<i>PKA1</i> encodes the major cAMP-dependent protein kinase catalytic subunit; attenuated in mammalian models	9.0 days ( <i>P</i> = 0.0007)
H99 <i>pka1</i> + <i>PKA1</i> (44)	Complementation of the <i>pka1</i> mutant with wild-type <i>PKA1</i> restored virulence in rabbits and mice	5.0 days (no difference)
H99 <i>ras1</i> (46)	<i>ras1</i> mutant is avirulent in an animal model of cryptococcal meningitis	>10 days ( <i>P</i> < 0.0001)
H99 <i>ras1</i> + <i>RAS1</i> (46)	Complementation of the <i>ras1</i> mutant with wild-type <i>RAS1</i> restored virulence in rabbits	5.0 days (no difference)
H99 <i>pkrl</i> (44)	<i>PKR1</i> encodes the protein kinase A (PKA) regulatory subunit; in mice, a <i>pkrl</i> mutant overproduces capsule and is hypervirulent	3.5 days ( <i>P</i> = 0.0001)
H99 M001 (49, 50)	Adenine auxotroph lacking phosphoribosyl-aminimidazole; avirulent when inoculated intrathecally into immunosuppressed rabbits	>10 days ( <i>P</i> < 0.0001)
H99 M001.1c (49)	Prototrophic transformant of M001 that has received a cloned <i>ADE2</i> cDNA; virulence was completely restored in rabbit model	6.0 days (no difference)
H99 <i>pMfa1::GFP</i> (51)	Promoter from the <i>C. neoformans</i> $\alpha$ pheromone gene fused to <i>GFP</i>	5.5 days (no difference compared to H99)
H99 <i>cap59</i> (52)	<i>CAP59</i> is essential for capsule formation	6.0 days in rich media (no difference); in minimal media, LT <sub>50</sub> = 5.0 days compared to 3.5 days for H99 ( <i>P</i> < 0.0001)
JEC20/JEC21 (48, 53, 54)	A pair of congenic <i>MATa</i> (JEC20) and <i>MAT<math>\alpha</math></i> (JEC21) strains	LT <sub>50</sub> = 9.5 days for JEC20; LT <sub>50</sub> = 5.5 for JEC21 ( <i>P</i> = 0.0043; JEC20 compared to JEC21)
ATCC #208820 (2e-tuc4) (40)	Serotype D; <i>MAT<math>\alpha</math></i> <i>CNLAC1</i> /laccase positive	6.0 days (LT <sub>50</sub> = 2.0 days in the presence of dopamine)
ATCC #208819 (2e-tu4) (40)	Serotype D; <i>MAT<math>\alpha</math></i> <i>cnlac1</i> /laccase negative	6.0 days (no difference from 2e-tuc4 in the absence of dopamine; LT <sub>50</sub> = 4.0 days and <i>P</i> < 0.0001 compared to 2e-tuc4 in the presence of dopamine)

infection. These data suggest that important aspects of *C. neoformans* pathogenesis in humans (and possibly that of other fungal pathogens) can be modeled by using the simple invertebrate nematode *C. elegans* as an experimental host.

## Materials and Methods

**Strains and Media.** The *C. neoformans* strains used in these experiments are summarized in Table 1 or described in the text. *C. laurentii* strains ATCC#18803, ATCC#66036, and ATCC#76483 and *C. kuetzingii* strain ATCC#42276 were obtained from the American Type Culture Collection (ATCC). Yeast cultures were maintained on yeast extract/peptone/dextrose (YPD; Difco) agar. The standard *C. elegans* strain N2 Bristol (55) was maintained at 15°C and propagated on *E. coli* strain OP50 by using established procedures (6, 55, 56).

***C. elegans* Killing Assays.** Yeast lawns were grown for *C. elegans* killing assays as follows: *C. neoformans* strains were inoculated into 2 ml of YPD and grown at 28°C for 48 h; 10  $\mu$ l of the culture was spread on 35-mm tissue-culture plates (Falcon) containing brain heart infusion (BHI) agar (Difco). The plates were incubated at 28°C overnight. *C. laurentii* and *C. kuetzingii* lawns for killing assays were prepared similarly except that growth was at 25°C. Ampicillin (100  $\mu$ g/ml) or gentamicin (25–50  $\mu$ g/ml) was added to the medium to selectively prevent growth of *E. coli* OP50 carried over on transfer of worms to the yeast-containing plates. For melanization studies, dopamine (100  $\mu$ g/ml) was added to the BHI medium. For some killing studies, standard nematode growth medium [NGM, minimal medium containing NaCl, agar, peptone, cholesterol, CaCl<sub>2</sub>, MgSO<sub>4</sub>, and potassium phosphate (55)] supplemented with Synthetic Complete Supplement Mixture (SC Amino Acid Mixture, Qbiogene, Carlsbad, CA) was used instead of BHI.

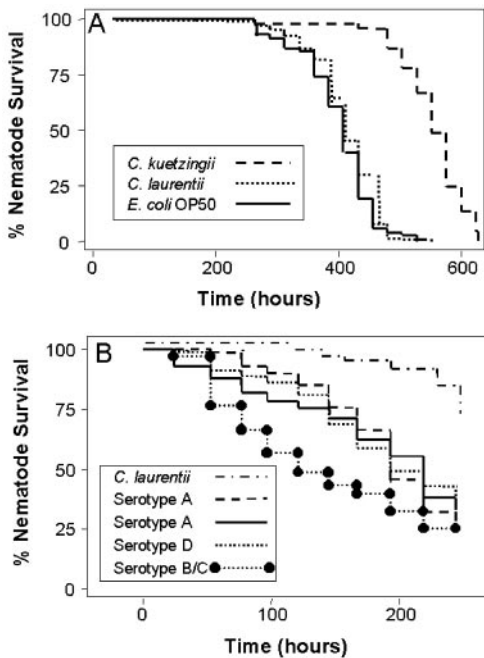
Between 40 and 50 *C. elegans* animals at the young adult developmental stage were transferred from a lawn of *E. coli* OP50

on NGM (55) to a lawn of the yeast to be tested on BHI medium, incubated at 25°C, and examined for viability at 12- to 24-h intervals with a dissecting microscope. Worms were considered dead when they did not respond to touch with a platinum wire pick. Each experimental condition was tested in duplicate or triplicate. Nematodes exposed to *C. laurentii* or *C. kuetzingii* were able to grow and produce progeny similarly to those growing on *E. coli* OP50. For these latter two yeasts, we transferred nematodes to new plates every 48 h to allow determination of survival of the original worms without the presence of progeny. Worms growing on *C. neoformans* did not produce any progeny.

Plotting of killing curves, calculation of LT<sub>50</sub> (time for half of the worms to die) and estimation of differences in survival (log-rank and Wilcoxon tests) with the Kaplan–Meier method were performed by using STATA 6 statistical software (Stata, College Station, TX). *P* values < 0.05 were considered statistically significant.

**Evaluation of the Life Span of *C. elegans*.** Evaluation of the life span of *C. elegans* when feeding on *E. coli* OP50, *C. kuetzingii*, or *C. laurentii* was carried out at 25°C, as described (56). In brief, 3-day-old nematodes at the L4 developmental stage maintained on *E. coli* OP50 on NGM were transferred to fresh NGM plates containing 100  $\mu$ g/ml 5-fluoro-2'-deoxyuridine (FdUrd; Sigma), which had been seeded with grown cultures of the bacterium or yeast under study. The animals remained on the FdUrd NGM plates for the remainder of their life spans; surviving nematodes were counted every day and dead ones were removed. Alternatively, nematode survival was evaluated on BHI plates. For this, nematodes were transferred to new plates every 24–48 h to allow determination of survival of the original worms without the presence of progeny.

**Microscopic Studies.** Nematodes were exposed to yeast on BHI plates for various times and then placed on a pad of 2% agarose



**Fig. 1.** (A) Life span of wild-type *C. elegans* N2 on NGM plates containing 5-fluoro-2'-deoxyuridine (FdUrd) feeding on lawns of *C. kuetzingii* ATCC#42276, *C. laurentii* ATCC#18803, and *E. coli* OP50.  $P < 0.0001$  for *E. coli* OP50 or *C. laurentii* compared with *C. kuetzingii*. Similar results were obtained with *C. laurentii* strains ATCC#66036 and ATCC#76483. (B) Survival of *C. elegans* N2 feeding on lawns of *C. neoformans* serotype A (ATCC#62067, ATCC#62068), serotype B/C (ATCC#34877), or serotype D (ATCC#36556), or on a lawn of *C. laurentii* ATCC#18803.  $P < 0.001$  for each of the *C. neoformans* strains compared with *C. laurentii*.

in a 5- $\mu$ l drop of 30 mM  $\text{NaN}_3$  in M9 medium. The worms were examined with an Axioplan2 microscope (Zeiss) fitted with Nomarski optics. For experiments involving cryptococcal strains containing the pYGF3 plasmid (57) expressing the synthetic GFP, the same methodology was used except that worms were viewed with a Leica TCS NT confocal spectrophotometer.

## Results

**Survival and Killing of *C. elegans* by Cryptococci.** To determine whether cryptococci can serve as a food source for *C. elegans*, and to determine whether pathogenic cryptococci can kill *C. elegans*, we provided *C. neoformans*, *C. laurentii*, and *C. kuetzingii* to wild-type N2 *C. elegans* as the sole source of food.

When L4 worms were transferred from lawns of *E. coli* OP50 to lawns of three different *C. laurentii* strains or a strain of *C. kuetzingii* on NGM or BHI medium containing ampicillin or gentamicin to prevent the growth of *E. coli* OP50, the nematodes appeared healthy and produced numbers of progeny similar to those of *E. coli*-fed worms. The *C. elegans* progeny produced when feeding exclusively on *C. laurentii* and *C. kuetzingii* for several generations were morphologically indistinguishable from *E. coli*-fed worms, and it appeared likely that *C. elegans* could be propagated indefinitely on *C. laurentii* or *C. kuetzingii*. In addition, *C. elegans* lived at least as long when feeding on *C. laurentii* or *C. kuetzingii* as when feeding on *E. coli* OP50. As shown in Fig. 1A, on NGM medium containing 5-fluoro-2'-deoxyuridine (FdUrd) to prevent the production of progeny (the standard assay for determining *C. elegans* longevity), the nematodes had the longest life span when feeding on *C. kuetzingii* [ $\text{LT}_{50}$  (time for half of the worms to die) = 23 days;  $P < 0.0001$  compared with worms feeding on *E. coli* OP50], whereas longevity on *C. laurentii* was similar to that on *E. coli* OP50 ( $\text{LT}_{50}$  = 17 days). As reported previously (6), we found that *C. elegans* died

prematurely when fed *E. coli* OP50 that had been grown on BHI medium compared with *E. coli* grown on NGM medium (ref. 6 and data not shown), suggesting that *E. coli* OP50 can be pathogenic to *C. elegans* when grown on the appropriate medium.

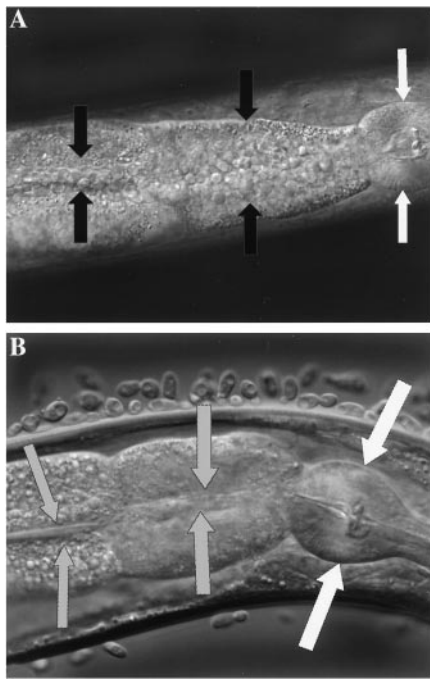
In contrast to *C. laurentii* and *C. kuetzingii*, all strains of *C. neoformans* that we tested killed *C. elegans*, and the nematodes were unable to make progeny. *C. elegans* killing was seen with human isolates of all known serotypes, including *C. neoformans* serotype A (ATCC#208821, which is also referred to as H99, ATCC#62067, and ATCC#62070), serotype B/C (ATCC#56695), and serotype D (ATCC#36556 and ATCC#24067) (Fig. 1B, Table 1, and data not shown). Environmental isolates of *C. neoformans* (ATCC#14116, ATCC#34870, ATCC#32308, ATCC#32045, and ATCC#62068) were also pathogenic to *C. elegans* (data not shown). Significant killing started at day 2 or 3 and most wild-type *C. neoformans* strains demonstrated an  $\text{LT}_{50}$  of 5–7 days with a range of 4–8.5 days (Fig. 1B, Table 1 and data not shown). *C. elegans* also died when exposed to mixed lawns of *C. neoformans* and *C. laurentii* (data not shown), indicating that *C. neoformans* actively kills *C. elegans* and excluding the trivial possibility that *C. neoformans* is simply not a nutritious food source for *C. elegans*.

In previous studies using *C. elegans* as a host to study bacterial pathogenesis, we observed that some bacterial pathogens induce the internal hatching of eggs in hermaphroditic worms, leading to matricide (1, 2, 6, 15). To exclude the possibility that killing of *C. elegans* by *C. neoformans* is caused solely by this matricidal effect (and not associated with an infectious-like process), we tested killing of *C. elegans glp-4* mutant worms. *C. elegans glp-4* animals have normal morphology and brood sizes at 15°C but do not make gonads and are unable to produce eggs at 25°C (58, 59). The  $\text{LT}_{50}$  of *glp-4* worms feeding on *C. neoformans* H99 at 25°C was  $\approx$  2 days longer compared with the  $\text{LT}_{50}$  for wild-type worms, but still substantially shorter than on *C. laurentii* or *C. kuetzingii*.

**Infection of *C. elegans* by Cryptococci.** Microscopic evaluation of nematodes exposed to *C. neoformans* H99 showed that the yeast cells accumulated inside an abnormally distended gastrointestinal tract (Fig. 2A). Similar results have been observed previously with *C. elegans* feeding on a variety of bacterial pathogens (2, 6, 13). Yeast cells were mostly concentrated at the area directly distal to the pharyngeal grinder (the organ that functions to disrupt ingested organisms). However, yeast cells could be seen throughout the nematode gastrointestinal tract. It is unlikely that the accumulation is solely due to proliferation of the yeast in the worm intestine, as the gastrointestinal tract was filled with *C. neoformans* within 6 h of the start of feeding. In contrast to *C. neoformans*, neither *C. laurentii* (Fig. 2B) nor *C. kuetzingii* cells (not shown) were seen inside nematodes feeding on these yeasts, and there was no dilation of the intestinal tract for at least 7 days. Of note, when we allowed nematodes to feed on a lawn of *C. neoformans* H99 and then, at different times, transferred them to lawns of *C. laurentii*, they were rescued from *C. neoformans* killing for each of the time points tested (up to 48 h). Moreover, the intestines of the rescued worms regained a normal-nondistended anatomy, no yeast cells could be observed in the intestines of the rescued worms, and no H99 cells could be cultured following disruption of the rescued worms (data not shown). These latter data suggest that *C. neoformans* strain H99 is not capable of permanently colonizing the intestine.

**Genes Associated with Pathogenesis in Mammals Cause Enhanced Killing of *C. elegans*.** Capsule growth and melanin production in *C. neoformans* are regulated by a G- $\alpha$  protein-cAMP-PKA (cAMP-dependent protein kinase A) signaling pathway. PKA is composed of catalytic and regulatory subunits encoded by the *PKA1* and *PKR1* genes, respectively (44). Strains in which *GPA1* (which encodes the G- $\alpha$  protein) or *PKA1* are disrupted display attenuated virulence in experimental murine cryptococcosis. On the other hand, a *C. neoformans* strain with a disruption in the



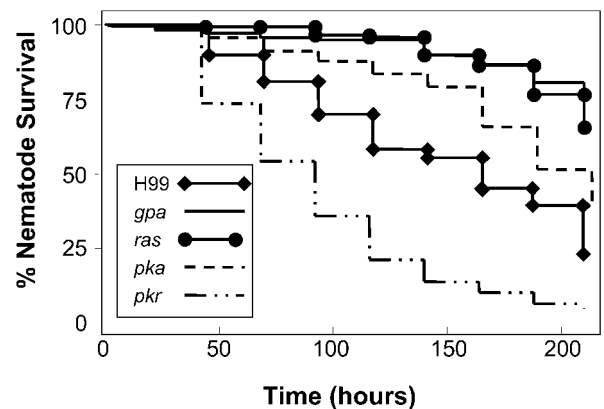


**Fig. 2.** *C. neoformans* but not *C. laurentii* accumulates in the gastrointestinal tract of *C. elegans*. (A) Intact yeast cells present in the distended gastrointestinal tract after feeding 24 h on *C. neoformans* strain H99. (B) No *C. laurentii* cells can be detected in the gastrointestinal tract after 24 h of feeding. The round structure (white arrows) is the pharyngeal grinder organ, which functions to disrupt ingested organisms. Black and gray arrows point to the intestinal lumen.

*PKR1* gene overproduces capsule and is hypervirulent in animal models (43, 44). As shown in Fig. 3 and Table 1, the *gpa1* and *pka1* mutants were significantly less virulent than the wild type, whereas the *pkrl* mutant was hypervirulent in the *C. elegans* model, similar to the results in mice. Reconstitution of a *Pka*<sup>+</sup> phenotype (by integrating the wild-type *PKA1* gene linked to the hygromycin B resistance gene into the mutant strain) that restored virulence in mammalian models (42–44), also restored virulence in the *C. elegans* model (Table 1).

The results described in the preceding paragraph suggest that capsule and/or melanization may play a role in *C. neoformans*-mediated *C. elegans* killing. In mammalian hosts, the *C. neoformans* polysaccharide capsule, which distinguishes *C. neoformans* from many other pathogenic fungi, protects against phagocytosis and killing by immune effector cells and blocks the production of cytokines and antigen presentation to T cells (20, 60, 61). To determine the role of capsule in *C. elegans* killing, we tested two different *C. neoformans* acapsular strains, 602 (ATCC#36555) and *cap59*, a derivative of H99 (52). Both 602 and *cap59* caused killing of *C. elegans* without accumulation in the nematode gastrointestinal tract (LT<sub>50</sub> = 5 and 6 days, respectively; Table 1 and data not shown), suggesting that neither capsule nor accumulation is necessary for nematode killing. In rich media (BHI), killing by *cap59* was similar to that caused by the wild-type parent H99 (LT<sub>50</sub> = 6 days). However, under conditions that promote capsule formation, such as growth in minimal media (NGM) (20, 60), killing by *cap59* was significantly slower compared with H99 (LT<sub>50</sub> = 3.5 and 5, respectively; *P* < 0.0001; Table 1). Moreover, lawns of heat killed H99 grown on rich media led to *C. elegans* killing with an LT<sub>50</sub> of 9 days, whereas nematodes survived at least 14 days when exposed to lawns of heat-killed 602 or *cap59* (data not shown). In summary, these results suggest that capsule plays a significant, but not essential, role in *C. neoformans*-mediated killing of *C. elegans*.

Melanization is also regulated by the G- $\alpha$  protein-cAMP-PKA



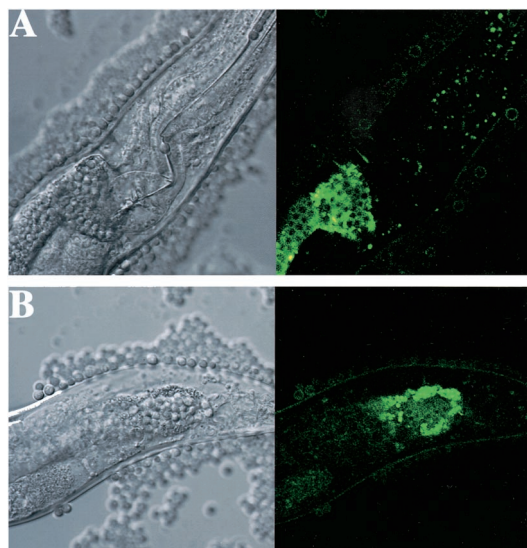
**Fig. 3.** *C. neoformans* virulence factors for mammalian infection also enhance killing of *C. elegans*. Survival of *C. elegans* N2 animals feeding on *C. neoformans* mutants with disruptions in the genes encoding the G protein-cAMP-PKA and the RAS1-controlled signal transduction cascades demonstrated hypovirulence (*gpa1*, *ras1*, and *pka1*) or hypervirulence (*pkrl*), similar to results in mammalian models. *P* < 0.001 for each of the mutants compared with the parental strain H99 (see Table 1).

signaling pathway. *C. neoformans* is often melanized in the environment (37), and melanization has been associated with protection against harsh environmental conditions such as UV radiation and extremes of temperature (62). Melanin is also a potent free radical scavenger and melanization has been shown to protect *C. neoformans* from macrophages and has been associated with virulence in murine studies (36–38, 62). Melanin is produced when laccase (encoded by *CNLAC1*) catalyzes the oxidation of dopamine to quinones, which then polymerize to form melanin (36). Two otherwise isogenic *CNLAC1* and *cnlac1* strains (2e-tuc4 and 2e-tu4, respectively) have been extensively studied (40). In the *C. elegans* model, in the presence of dopamine (100  $\mu$ g/ml), the laccase-positive strain that was able to synthesize melanin was significantly more virulent compared with the laccase-deficient strain. As expected, the two strains exhibited no difference in virulence when the experiment was carried out by using standard media in the absence of L-dopa (LT<sub>50</sub> = 6 days—no difference compared with the parent strain in the absence of dopamine). In the presence of dopamine, the LT<sub>50</sub> of the laccase-negative strain decreased to 4 days, whereas the LT<sub>50</sub> of the laccase-positive strain decreased to 2 days (*P* < 0.0001; Table 1).

In addition to the G- $\alpha$  protein-cAMP-PKA signaling pathway, another regulatory pathway associated with *C. neoformans* maintenance of infection in mammalian hosts involves RAS1-specific signaling cascades that are required for growth at high temperature (37°C) (46). In the *C. elegans* model, a *ras1* mutant that was previously found to be attenuated in mammalian models was significantly attenuated at 25°C compared with wild type; reconstitution of the Ras1<sup>+</sup> phenotype (by integrating the wild-type *RAS1* gene linked to the hygromycin B resistance gene into the *ras1* mutant strain) restored virulence (Table 1). These findings indicate that *RAS1* plays a role in *C. neoformans* beyond its role in promoting growth at 37°C.

The phosphoribosylaminoimidazole gene (*ADE2*) has been demonstrated to be essential for growth of *C. neoformans* in the cerebrospinal fluid, and an *ade2* auxotroph of H99 (M001) was avirulent in a cryptococcal meningitis model in corticosteroid-treated rabbits (49). In the *C. elegans* model, the LT<sub>50</sub> for killing by the M001 *ade2* mutant was >10 days, compared with 6 days for the H99 parents; virulence was completely restored by the reintroduction of the wild-type *ADE2* gene in the M001.1c strain (Table 1).

**Activation of Mating Type  $\alpha$  Gene During *C. elegans* Infection.** *C. neoformans* can reproduce asexually by budding or can form spores



**Fig. 4.** The *C. neoformans* *MF $\alpha$ 1* promoter is specifically expressed in the *C. elegans* intestine (magnification  $\times 40$ ). Fluorescent microscopy of wild-type *C. elegans* N2 after feeding for 3 days on *C. neoformans* H99 expressing GFP fused to the *MF $\alpha$ 1* promoter. Green fluorescence is seen in yeast cells inside the proximal (A) and the distal (B) end of the *C. elegans* intestine. There was no fluorescence associated with yeast cells outside the nematodes and no fluorescence was observed on day 1 or 2 of this experiment.

when cells of opposite mating types (MAT $\alpha$  and MAT $\alpha$ ) undergo conjugation. The MAT $\alpha$  mating type has been associated with increased virulence in animal models (41). Similarly, MAT $\alpha$  mutants lacking the MF $\alpha$  pheromone were subtly or modestly attenuated compared with wild type in a murine tail vein injection model (63). We found that the MAT $\alpha$  mating type was also more virulent than the MAT $\alpha$  mating type with respect to *C. elegans* killing (LT<sub>50</sub> = 5.5 days compared with 9.5 days;  $P = 0.0043$ ; Table 1). The GFP from the jellyfish *Aequorea victoriae* has been used as a reporter of gene expression in a variety of heterologous systems. Cormack *et al.* (57) developed a modified GFP protein (yEGFP3) that generates significantly more fluorescence than wild-type GFP in the fungi *Saccharomyces cerevisiae* and *Candida albicans*. Using this modified GFP, del Poeta and coworkers (51) showed that a *C. neoformans* mating type  $\alpha$ -specific reporter gene fusion *pMF $\alpha$ 1::GFP* (*pMF $\alpha$ 1* regulates production of MF $\alpha$  pheromone) was activated during the proliferative stage of infection in the rabbit CNS but not during earlier phases of infection. Similar to the results in the rabbit CNS, as shown in Fig. 4, we found that there was no *pMF $\alpha$ 1::GFP* expression in *C. neoformans* cells that were not ingested by nematodes, but that there was significant GFP expression in yeast cells within the nematode intestine by three days after ingestion. Although the *C. elegans* model has been used in the study of virulence by a number of pathogenic bacteria, this is the first direct evidence demonstrating that expression of a gene associated with virulence is temporally related to *C. elegans* killing.

## Discussion

In this paper, we report the observations that *C. elegans* can feed on yeasts, that a human pathogenic yeast can kill *C. elegans*, and that fungal virulence factors important for mammalian pathogenesis also play a role in *C. elegans* killing, findings that validate the use of *C. elegans* as a model host to study fungal pathogenesis. Similar to the hypothesis proposed by Steenbergen *et al.* (18) as a consequence of their studies examining phagocytosis and replication of *C. neoformans* in *A. castellanii* (18), mammalian pathogenesis of *C. neoformans* may be a consequence of adaptations that have

evolved during the interaction of *C. neoformans* with environmental predators such as free-living nematodes and amoebae.

***C. elegans* Can Feed on Yeasts.** Despite the relatively large size of yeast cells compared with bacterial cells, it appears that *C. elegans* can use at least two different yeasts, *C. laurentii* and *C. kuetzingii*, as a sole source of food, producing the same number of healthy progeny as when feeding on *E. coli* strain OP50, the standard laboratory food source. Indeed, *C. kuetzingii* may be even more healthful than *E. coli* because the longevity of the worms is significantly longer when feeding on *C. kuetzingii* than on *E. coli*. This latter observation and other unpublished data from our laboratory (D. Garsin, J. Villanueva, J. Begun, C. Sifri, D. Kim, G. Ruvkun, and F.M.A., in preparation) indicates that *E. coli* OP50 may be mildly pathogenic to *C. elegans*.

***C. neoformans* Kills *C. elegans*.** In contrast to *C. laurentii* and *C. kuetzingii*, the human pathogenic yeast, *C. neoformans*, infects and kills *C. elegans*. Ecological studies have examined the interactions between fungi and free-living rhabditid nematodes (64), but only in the case of the endoparasitic fungus *Drechmeria coniospora* has a model of infection with *C. elegans* been established (65). Infection of *C. elegans* by *D. coniospora* starts by the adhesion of fungal spores to the head and is followed by the extension of hyphal processes into the nematodes (65). In contrast to *D. coniospora*, the mechanism by which *C. neoformans* accumulates within the *C. elegans* intestine and kills the worms is not immediately apparent from the studies reported here.

In previous studies, our laboratory and others have shown that the Gram-negative human pathogens *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Burkholderia pseudomallei*, and Gram-positive bacteria such as *Enterococcus faecalis*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* also kill adult *C. elegans* (1–15). The *C. neoformans*/*C. elegans* model shares a variety of features with these *C. elegans* models involving bacterial pathogens. For example, as in the case of *C. neoformans*, accumulation within the gastrointestinal tract is associated with killing of *C. elegans* in most of the bacterial models. It is very unlikely that *C. neoformans*-mediated “killing” is a consequence of *C. neoformans* being a nonnutritious food source. Mixed lawns of *C. neoformans* and *C. laurentii* also killed and >50% of the nematodes exposed to the *C. neoformans* adenine auxotroph M001 survived for more than 10 days. Although we cannot formally rule out the possibility that accumulation *per se* of *C. neoformans* in the *C. elegans* intestine and the resulting distention of the intestine is sufficient for killing, we have observed previously that *Enterococcus faecium* accumulates to very high levels causing intestinal distention but does not kill *C. elegans* (6). In any case, *C. neoformans* acapsular strains (such as *cap59*) also kill *C. elegans* without accumulation inside the nematode intestine. A similar phenomenon was observed in the case of the *P. aeruginosa*/*C. elegans* model, where *P. aeruginosa* also killed without accumulation by a toxin-mediated process when grown on particular media (13). Killing by the acapsular *C. neoformans* strains could also be attributable to toxins produced by the yeast or by components of the yeast cell (such as parts of the cell wall) that are generated as the nematode grinds the yeast cells. It is not clear why heat-killed *C. neoformans* strains also killed, but this could also be attributable to the production of toxins or cell-wall fragments.

***C. neoformans* Virulence Factors.** An important feature of the *C. elegans*/bacterial models of pathogenesis developed previously is that a variety of bacterial virulence factors involved in mammalian pathogenesis were also shown to be involved in nematode killing. We provide several examples in this paper that demonstrate that this is also the case for *C. neoformans*-mediated killing of *C. elegans*.

One of the most extensively studied *C. neoformans* virulence factors is its polysaccharide capsule (30, 35). In the *C. elegans* model,



capsule may be sufficient for nematode killing because heat-killed capsular strains killed much more efficiently than heat-killed acapsular strains. On the other hand, capsule was not absolutely necessary for virulence as the acapsular strains *cap59* and 602 also killed. Moreover, in rich media where relatively little capsule is made, there was no observable difference in nematode killing by *cap59* and its isogenic wild-type parent. This differentiates the *C. elegans* and the *A. castellanii* pathogenesis systems, because capsule was necessary for *C. neoformans* virulence in the free-living amoebae (18). On the other hand, although the *C. neoformans cap59* mutant is acapsular, it still may synthesize certain components of the capsule that are toxic. Of note is that *C. laurentii* also forms capsule, but it is rarely identified from clinical specimens and was not pathogenic to nematodes. The lack of pathogenicity of *C. laurentii* has been attributed to the inability of most strains to grow at 37°C. However, in the *C. elegans* model, *C. laurentii* was non-pathogenic at 25°C, suggesting that it may lack additional virulence traits unique to *C. neoformans*.

In addition to the *C. neoformans* capsule, we found extensive correlation between *C. neoformans* factors involved in both *C. elegans* and mammalian pathogenesis. *C. neoformans* mutants with disruptions in the genes associated with two signal transduction systems demonstrated hypo- (*GPA1*, *PKA1*, and *RAS1*) or hyper-virulence (*PKR1*), as in established mammalian models. In the presence of the melanin substrate L-dopamine, a laccase-positive strain was significantly more virulent than an isogenic laccase-deficient strain. Also, the *C. neoformans ADE2* gene is important for *C. elegans* killing, as in established mammalian models. A final example of the similarity between mammalian models and the *C. elegans* model of *C. neoformans* pathogenesis is the fact that the *C.*

*neoformans MFA1* gene, which is specifically activated during pathogenesis in a mammalian host (51), is similarly activated during infection of the *C. elegans* intestine. It is likely that the conditions inside the nematode gastrointestinal tract stimulate this pathway and it would be interesting to identify both *C. neoformans* and *C. elegans* mutants that failed to induce the *MFA1-GFP* reporter gene.

Previously, our laboratory and others have shown that *C. elegans* can be used to identify and study bacterial virulence factors relevant for mammalian pathogenesis by screening individual clones from bacterial mutant libraries for clones that exhibited impaired *C. elegans* killing (2, 6, 10–12). The results reported here suggest that a similar strategy could be used to identify *C. neoformans* virulence factors important for mammalian pathogenesis, irrespective of the fact that there may be many unique features of the *C. elegans/C. neoformans* model that are not directly relevant to mammalian pathogenesis or that particular virulence factors may play a more significant role in one of the models compared with the other.

In conclusion, the interaction of *C. elegans* with the yeast *C. neoformans* involves a number of genes that are also important during the host/pathogen interaction during mammalian infection. Identification of new *C. neoformans* virulence factors using this model may lead to new targets for antifungal therapies as well as a deeper understanding of the host–fungus interaction.

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- Aballay, A. & Ausubel, F. M. (2002) *Curr. Opin. Microbiol.* **5**, 97–101.
- Aballay, A., Yorgey, P. & Ausubel, F. M. (2000) *Curr. Biol.* **10**, 1539–1542.
- Aballay, A. & Ausubel, F. M. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 2735–2739.
- Choi, J. Y., Sifri, C. D., Goumnerov, B. C., Rahme, L. G., Ausubel, F. M. & Calderwood, S. B. (2002) *J. Bacteriol.* **184**, 952–961.
- Darby, C., Cosma, C. L., Thomas, J. H. & Manoil, C. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 15202–15207.
- Garsin, D. A., Sifri, C. D., Mylonakis, E., Qin, X., Singh, K. V., Murray, B. E., Calderwood, S. B. & Ausubel, F. M. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 10892–10897.
- Kurz, C. L. & Ewbank, J. J. (2000) *Trends Microbiol.* **8**, 142–144.
- Labrousse, A., Chauvet, S., Couillault, C., Kurz, C. L. & Ewbank, J. J. (2000) *Curr. Biol.* **10**, 1543–1545.
- Plotnikova, J. M., Rahme, L. G. & Ausubel, F. M. (2000) *Plant Physiol.* **124**, 1766–1774.
- Rahme, L. G., Stevens, E. J., Wolford, S. F., Shao, J., Tompkins, R. G. & Ausubel, F. M. (1995) *Science* **268**, 1899–1902.
- Rahme, L. G., Tan, M.-W., Le, L., Wong, S. M., Tompkins, R. G., Calderwood, S. B. & Ausubel, F. M. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 13245–13250.
- Rahme, L. G., Ausubel, F. M., Cao, H., Drenkard, E., Goumnerov, B. C., Lau, G. W., Mahajan-Miklos, S., Plotnikova, J., Tan, M.-W., Tsongalis, J., et al. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 8815–8821.
- Tan, M.-W., Mahajan-Miklos, S. & Ausubel, F. M. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 715–720.
- Tan, M.-W., Rahme, L. G., Sternberg, J. A., Tompkins, R. G. & Ausubel, F. M. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 2408–2413.
- Tan, M.-W. & Ausubel, F. M. (2000) *Curr. Opin. Microbiol.* **3**, 29–34.
- Kim, D. H., Feinbaum, R., Alloing, G., Emerson, F. E., Garsin, D. A., Inoue, H., Tanaka-Hino, M., Hisamoto, N., Matsumoto, K., Tan, M.-W. & Ausubel, F. M. (2002) *Science* **297**, 623–626.
- Mallo, G. V., Kurz, C. L., Couillault, C., Pujol, N., Granjeaud, S., Kohara, Y. & Ewbank, J. (2002) *Curr. Biol.* **12**, 1209–1214.
- Steenbergen, J. N., Shuman, H. A. & Casadevall, A. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 15245–15250.
- Levitz, S. M. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 14760–14762.
- Casadevall, A. & Perfect, J. R. (1998) *Cryptococcus neoformans* (Am. Soc. Microbiol., Washington, DC).
- Marty, F. & Mylonakis, E. (2002) *Exp. Opin. Pharmacother.* **3**, 91–102.
- Mylonakis, E., Rich, J., Skolnik, P. R., De Orchis, D. F. & Flanagan, T. (1997) *Medicine (Baltimore)* **76**, 249–255.
- Mylonakis, E., Barlam, T. F., Flanagan, T. & Rich, J. D. (1998) *Chest* **114**, 251–262.
- Mylonakis, E., Paliou, M., Sax, P. E., Skolnik, P. R., Baron, M. J. & Rich, J. D. (2000) *Medicine (Baltimore)* **79**, 269–280.
- Pappas, P. G., Perfect, J. R., Cloud, G. A., Larsen, R. A., Pankey, G. A., Lancaster, D. J., Henderson, H., Kauffman, C. A., Haas, D. W., Saccante, M., et al. (2001) *Clin. Infect. Dis.* **33**, 690–699.
- Thomas, C. J., Lee, J. Y., Conn, L. A., Bradley, M. E., Gillespie, R. W., Dill, S. R., Pinner, R. W. & Pappas, P. G. (1998) *Ann. Epidemiol.* **8**, 212–216.
- Vandenkoornhuysem, P., Baldauf, S. L., Leyval, C., Straczek, J. & Young, J. P. (2002) *Science* **295**, 2051.
- Kwon-Chung, K. J. (1976) *Mycologia* **68**, 821–833.
- Gumbo, T., Kadzirange, G., Mielke, J., Gangaidzo, I. T. & Hakim, J. G. (2002) *Pediatr. Infect. Dis. J.* **21**, 54–56.
- Chang, Y. C. & Kwon-Chung, K. J. (1994) *Mol. Cell. Biol.* **14**, 4912–4919.
- Edman, J. C. & Kwon-Chung, K. J. (1990) *Mol. Cell. Biol.* **10**, 4538–4544.
- Hua, J., Meyer, J. D. & Lodge, J. K. (2000) *Clin. Diagn. Lab. Immunol.* **7**, 125–128.
- Lodge, J. K., Jackson-Machelski, E., Toffaletti, D. L., Perfect, J. R. & Gordon, J. I. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 12008–12012.
- Toffaletti, D. L., Rude, T. H., Johnston, S. A., Durack, D. T. & Perfect, J. R. (1993) *J. Bacteriol.* **175**, 1405–1411.
- Perfect, J. R., Wong, B., Chang, Y. C., Kwon-Chung, K. J. & Williamson, P. R. (1998) *Med. Mycol.* **36**, Suppl. 1, 79–86.
- Salas, S. D., Bennett, J. E., Kwon-Chung, K. J., Perfect, J. R. & Williamson, P. R. (1996) *J. Exp. Med.* **184**, 377–386.
- Casadevall, A., Rosas, A. L. & Nosanchuk, J. D. (2000) *Curr. Opin. Microbiol.* **3**, 354–358.
- Wang, Y., Aisen, P. & Casadevall, A. (1995) *Infect. Immun.* **63**, 3131–3136.
- Jacobson, E. S. (2000) *Clin. Microbiol. Rev.* **13**, 708–717.
- Williamson, P. R., Wakamatsu, K. & Ito, S. (1998) *J. Bacteriol.* **180**, 1570–1572.
- Kwon-Chung, K. J., Edman, J. C. & Wickes, B. L. (1992) *Infect. Immun.* **60**, 602–605.
- Alspaugh, J. A., Perfect, J. R. & Heitman, J. (1997) *Genes Dev.* **11**, 3206–3217.
- D'Souza, C. A. & Heitman, J. (2001) *FEMS Microbiol. Rev.* **25**, 349–364.
- D'Souza, C. A., Alspaugh, J. A., Yue, C., Harashima, T., Cox, G. M., Perfect, J. R. & Heitman, J. (2001) *Mol. Cell. Biol.* **21**, 3179–3191.
- Lengeler, K. B., Davidson, R. C., D'Souza, C. A., Harashima, T., Shen, W. C., Wang, P., Pan, X., Waugh, M. & Heitman, J. (2000) *Microbiol. Mol. Biol. Rev.* **64**, 746–785.
- Alspaugh, J. A., Cavallo, L. M., Perfect, J. R. & Heitman, J. (2000) *Mol. Microbiol.* **36**, 352–365.
- Waugh, M. S., Nichols, C. B., DeCesare, C. M., Cox, G. M., Heitman, J. & Alspaugh, J. A. (2002) *Microbiology* **148**, 191–201.
- Heitman, J., Casadevall, A., Lodge, J. K. & Perfect, J. R. (1999) *Mycopathologia* **148**, 1–7.
- Perfect, J. R., Toffaletti, D. L. & Rude, T. H. (1993) *Infect. Immun.* **61**, 4446–4451.
- Sudarshan, S., Davidson, R. C., Heitman, J. & Alspaugh, J. A. (1999) *Fungal Genet. Biol.* **27**, 36–48.
- del Poeta, M., Toffaletti, D. L., Rude, T. H., Sparks, S. D., Heitman, J. & Perfect, J. R. (1999) *Infect. Immun.* **67**, 1812–1820.
- Nelson, R. T., Hua, J., Pryor, B. & Lodge, J. K. (2001) *Genetics* **157**, 935–947.
- Moore, T. D. & Edman, J. C. (1993) *Mol. Cell. Biol.* **13**, 1962–1970.
- Karos, M., Chang, Y. C., McClelland, C. M., Clarke, D. L., Fu, J., Wickes, B. L. & Kwon-Chung, K. J. (2000) *J. Bacteriol.* **182**, 6222–6227.
- Brenner, S. (1974) *Genetics* **77**, 71–94.
- Honda, S., Ishii, N., Suzuki, K. & Matsuo, M. (1993) *J. Gerontol.* **48**, B57–B61.
- Cormack, B. J., Bertram, G., Egerton, M., Gow, N. A., Falkow, S. & Brown, A. J. (1997) *Microbiology* **143**, 303–311.
- Beanan, M. J. & Strome, S. (1992) *Development (Cambridge, U.K.)* **116**, 755–766.
- Roussell, D. L. & Bennett, K. L. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 9300–9304.
- Doering, T. L. (2000) *Trends Microbiol.* **8**, 547–553.
- Feldmesser, M., Riveria, J., Kress, Y., Kozel, T. R. & Casadevall, A. (2000) *Infect. Immun.* **68**, 3642–3650.
- Rosas, A. L. & Casadevall, A. (2001) *Mycopathologia* **151**, 53–56.
- Shen, W. C., Davidson, R. C., Cox, G. M. & Heitman, J. (2002) *Eukaryot. Cell* **1**, 366–377.
- Mendoza De Givès, P. M., Davies, K. G., Clark, S. J. & Behnke, J. M. (1999) *Parasitology* **119**, 95–104.
- Jansson, H. B., Jeyapakash, A. & Zuckerman, B. M. (1985) *Appl. Environ. Microbiol.* **49**, 552–555.